

# Calcium-independent activation of skeletal muscle fibers by a modified form of cardiac troponin C

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**ABSTRACT** A conformational change accompanying  $\text{Ca}^{2+}$  binding to troponin C (TnC) constitutes the initial event in contractile regulation of vertebrate striated muscle. We replaced endogenous TnC in single skinned fibers from rabbit psoas muscle with a modified form of cardiac TnC (cTnC) which, unlike native cTnC, probably contains an intramolecular disulfide bond. We found that such activating TnC (aTnC) enables force generation and shortening in the absence of calcium. With aTnC, both force and shortening velocity were the same at pCa 9.2 and pCa 4.0. aTnC could not be extracted under conditions which resulted in extraction of endogenous TnC. Thus, aTnC provides a stable model for structural studies of a calcium binding protein in the active conformation as well as a useful tool for physiological studies on the primary and secondary effects of  $\text{Ca}^{2+}$  on the molecular kinetics of muscle contraction.

## INTRODUCTION

Vertebrate striated muscle contraction is regulated through  $\text{Ca}^{2+}$  binding to troponin, a protein complex on the thin, actin-containing filaments (1). The troponin regulatory complex consists of three subunits: TnC, which binds calcium, troponin I (TnI), an inhibitory subunit, and troponin T (TnT), which interacts strongly with tropomyosin (Tm). In the absence of  $\text{Ca}^{2+}$ , troponin and Tm inhibit strong interactions between actin and the thick filament protein myosin.  $\text{Ca}^{2+}$  binding to TnC changes its conformation, altering interactions with TnI. Ultimately the interaction between Tm and actin is altered to permit strong interactions between myosin and actin (1–4). Although the major action of  $\text{Ca}^{2+}$  is to initiate contraction in vertebrate striated muscle, other possible roles have been suggested for  $\text{Ca}^{2+}$ , such as modification of actomyosin kinetics by binding to myosin regulatory light chain or other proteins (5).

TnC contains four EF-hand divalent cation binding sites (6). In skeletal muscle, the two  $\text{NH}_2$ -terminal sites (I and II) bind  $\text{Ca}^{2+}$  specifically with relatively low affinity ( $K = 3 \times 10^5 \text{ M}^{-1}$ ) and are the regulatory sites; the two  $\text{COOH}$ -terminal sites (III and IV) have a relatively higher affinity for  $\text{Ca}^{2+}$  ( $K = 2 \times 10^7 \text{ M}^{-1}$ ), but also bind  $\text{Mg}^{2+}$  ( $K = 2 \times 10^3 \text{ M}^{-1}$ ), and thus are occupied by divalent cations under physiological conditions (7). In cardiac muscle, however, regulatory site I does not bind divalent cations and is not involved in regulation of contraction (7, 8). The high-resolution x-ray crystal structure of skeletal TnC (sTnC) has been determined with calcium bound only at sites III and IV (9, 10). In this structure, the  $\text{NH}_2$ -terminal and  $\text{COOH}$ -terminal domains are linked by an elongated  $\alpha$ -helix.

Structural comparison between the  $\text{NH}_2$ -terminal and  $\text{COOH}$ -terminal domains of sTnC led to the proposal that  $\text{Ca}^{2+}$  binding to sites I and II induces movement of the B and C helices away from the central axis of the molecule, enabling increased interaction of TnC with TnI (6, 11, 12). Recent observations with sTnC are consistent with this model. The ability of sTnC to regulate contractile activity and bind calcium was decreased when the D helix of the linker region was either cross-linked to the connecting loop between helices B and C (13), or connected by a salt bridge to the C helix flanking regulatory site II (14). However, this model has not been tested rigorously since there has not been a stable model of TnC in the active conformation.

Native cTnC contains two Cys residues (Cys 35 and Cys 84) (15). By homology with the structure of sTnC, Cys 35 of cTnC is expected to be located in the EF-hand domain of the nonfunctional site I and Cys 84 in the D helix of the linker region. Formation of an intramolecular disulfide bond between these cysteines in cTnC has been proposed to induce structural changes around site II which are similar to those which occur in the presence of  $\text{Ca}^{2+}$  (16). In this report, we prepared such a stable activating form of TnC (aTnC) and demonstrated its ability to activate force and shortening of skinned fibers in the absence of  $\text{Ca}^{2+}$ .

## MATERIALS AND METHODS

Single glycerinated fibers were prepared from rabbit psoas muscle according to Chase and Kushmerick (17). We chose this muscle (a) because of the detailed mechanochemical information already available regarding rabbit fast skeletal muscle proteins, (b) because we have developed methods for maintaining structural and mechanical stability of single skinned psoas fibers during prolonged activations (17, 18), and (c) because of the interchangeability of skeletal and cardiac TnC isoforms (5). Mechanical measurements were made as previously described (17). Sarcomere length (SL) was set to 2.5–2.6  $\mu\text{m}$  using laser diffraction and was monitored continuously (18). Throughout the experiment, the fiber was periodically shortened (4 fiber lengths  $\cdot \text{s}^{-1}$ ;

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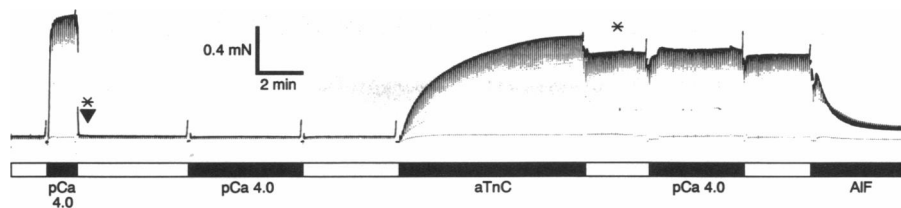


FIGURE 1 Force recorded during an experiment in which aTnC was substituted for endogenous sTnC in a single skinned fiber from rabbit psoas. The record is discontinuous at the times indicated by (\*). Time periods during which the fiber was exposed to  $\text{Ca}^{2+}$  (pCa 4.0) or other conditions are indicated below the force record as a solid line; the solution was pCa 9.2 elsewhere (see Methods). Force transients occur every 5 s due to ramp release/restretch cycles (see Methods). First, the fiber was activated in pCa 4.0 solution and relaxed to obtain a control value for maximum  $\text{Ca}^{2+}$  activated force. At the inverted triangle, the fiber was exposed to TnC extracting solution (see Methods) and subsequently tested for completeness of extraction by verifying the absence of  $\text{Ca}^{2+}$  activated force. The fiber was then reconstituted with aTnC ( $\sim 0.22 \text{ mg} \cdot \text{ml}^{-1}$  in pCa 9.2 solution) where indicated below the force record. After achievement of steady force, the fiber was transferred to relaxing solution (pCa 9.2) without TnC; force was higher in the aTnC reconstituting solution due to lack of CK in that solution only. At pCa 4.0, little or no increase in force was observed above that found in relaxing solution. Finally, force was inhibited with aluminofluoride (1 mM aluminum, 10 mM fluoride, pCa 9.2) where indicated below the force trace (AIF).

$\text{FL} \cdot \text{s}^{-1}$ ), which decreased force to the baseline, followed by restretch to the initial length; this procedure allowed sarcomere homogeneity to be maintained during prolonged activation (17–20).

Rabbit ventricular cTnC was prepared according to Potter (21). Protein concentration was determined by absorbance using an extinction coefficient of  $300 \text{ cm}^2 \cdot \text{g}^{-1}$  at 275 nm (22) and 18,459 MW (15). aTnC was prepared from fresh cTnC by dialysis for 3 days at  $22^\circ\text{C}$  against a solution containing 90 mM KCl, 2 mM [ethylenbis(oxyethylenitrilo)]tetraacetic acid (EGTA), 2 mM ethylenedinitrilotetraacetic acid (EDTA), 50  $\mu\text{M}$  1,10 phenanthroline, 10 mM 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.0. When incorporated into fibers, aTnC prepared in this manner exhibited 50–100%  $\text{Ca}^{2+}$ -insensitivity, as judged by force production. To test for reversible disulfide bond formation in aTnC, the isolated protein was dialyzed against 6 M urea, 20 mM 2-[N-morpholino]ethanesulfonic acid (MES), 1 mM EGTA, 5 mM dithiothreitol (DTT), pH 6.0, followed by dialysis against 90 mM KCl, 10 mM MOPS, 2 mM EGTA, 1 mM DTT, pH 7.0, and finally against pCa 9.2 solution.

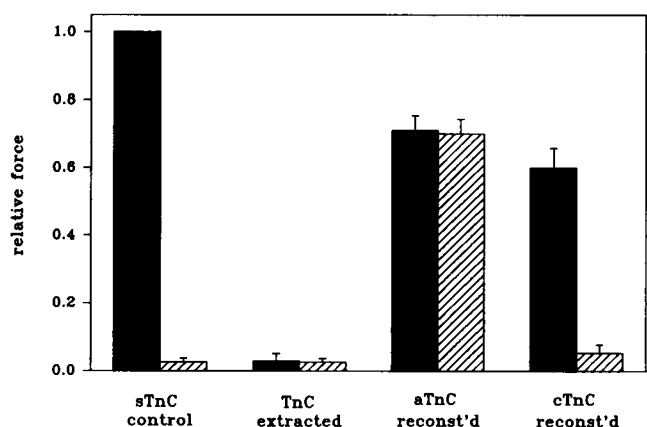


FIGURE 2 Force measured at pCa 4.0 (solid) and pCa 9.2 (hatched) for 18 single fibers from rabbit psoas muscle under control conditions with native sTnC, following complete extraction of TnC (5), and following reconstitution with either aTnC ( $N = 14$  fibers tested with a single preparation of aTnC which exhibited little or no  $\text{Ca}^{2+}$  sensitive component of force) or native cTnC ( $N = 4$ ). All force values were normalized to that obtained at pCa 4.0 for the same fiber prior to extraction; maximum  $\text{Ca}^{2+}$  activated force ( $100\%$ ) was  $285 \pm 91 \text{ mN} \cdot \text{mm}^{-2}$  ( $N = 18$ ).

The number of free cysteine residues per molecule was determined using the standard SH reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Prior to reaction with DTNB, the protein was treated with 100  $\mu\text{M}$  DTT under non-denaturing conditions; DTT was removed by ultrafiltration (Centricon-10 microconcentrator; Amicon, Beverly, MA). The reaction was carried out at  $20^\circ\text{C}$  with 4–10  $\mu\text{M}$  TnC, 1 mM DTNB, 6 M urea, 90 mM KCl, 10 mM MOPS, 2 mM EGTA and pH 7.0. Free cysteine concentration was obtained after 45 min by absorbance at 412 nm using a molar extinction coefficient of  $13,600 \text{ cm}^2 \cdot \text{M}^{-1}$ .

All solutions for physiological measurements contained 110 mM  $\text{K}^+$ , 20 mM  $\text{Na}^+$ , 12 mM EGTA, 15 mM phosphocreatine (PCr), 5 mM Mg-adenosine 5'-triphosphate (MgATP), 1 mM  $\text{Mg}^{2+}$ , 250 units  $\cdot \text{ml}^{-1}$  creatine phosphokinase (CK), 4% (wt/vol) Dextran T-500, and pH 7.0 at  $12^\circ\text{C}$ ; propionate was the major anion. The  $\text{Ca}^{2+}$  level (expressed as  $\text{pCa} = -\log [\text{Ca}^{2+}]$ ) was altered by varying the amount of  $\text{Ca}^{2+}$  (propionate) $_2$  (23). Endogenous sTnC was extracted from fibers in a solution containing 5 mM EDTA and 20 mM 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), pH 7.2, a modification of the method of Cox et al. (24), with the addition of 500  $\mu\text{M}$  trifluoroperazine (25). To accomplish complete extraction, fibers were incubated in the extracting solution for 20 min at  $12^\circ\text{C}$ .

Subsequent to mechanical measurements, fibers were removed from the apparatus and stored, along with control segments, at  $-20^\circ\text{C}$  for up to 2 weeks. Fiber extracts were analyzed for protein content by Na-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide) according to Giulian et al. (26) with minor modifications. Protein bands on the gels were visualized by silver staining.

Values are given as mean  $\pm$  S.D.. Statistical comparisons ( $t$ -tests) were performed using SigmaPlot 4.1 (Jandel Scientific, Corte Madera, CA).

## RESULTS AND DISCUSSION

We tested the ability of aTnC to activate skinned (detergent-treated, hyperpermeable) single cells from rabbit psoas. Extraction of native sTnC (see Methods) was judged to be complete because the fibers did not respond to  $\text{Ca}^{2+}$  activating solutions (Figs. 1, 2). Furthermore, SDS-PAGE of extracted single fibers revealed that sTnC was absent and that proteins other than sTnC were not affected by the procedure (Fig. 3A). Subsequent to extraction of sTnC, reconstitution with aTnC led to progressively increasing force in the absence of  $\text{Ca}^{2+}$  (Fig.

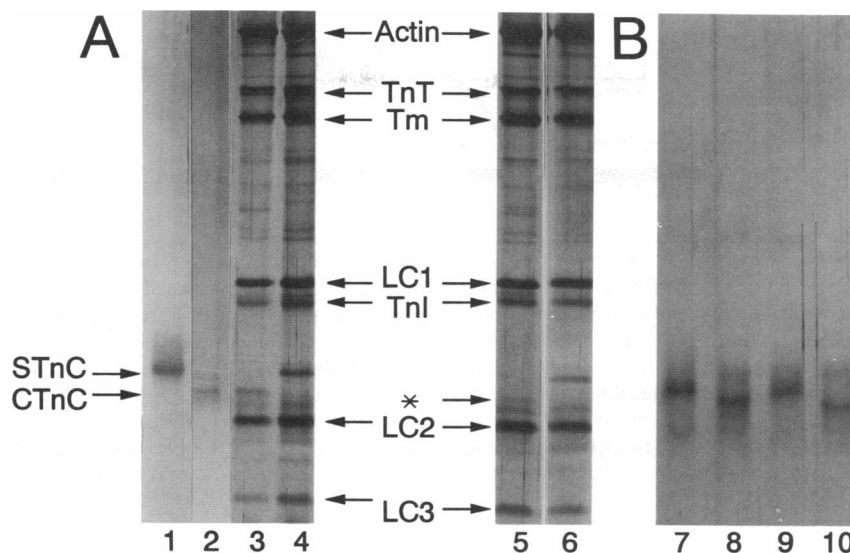


FIGURE 3 SDS-PAGE of purified TnC's and single muscle fiber segments. (A) Purified sTnC (lane 1) and cTnC (lane 2) from rabbit. Rabbit psoas muscle fibers from which sTnC was completely extracted followed by reconstitution with either native cTnC (lane 3) or aTnC (\*, lane 5); corresponding control segments are shown in lanes 4 and 6, respectively. LC1, LC2, and LC3 are myosin light chains 1, 2 (regulatory light chain), and 3, respectively. (B) SDS-PAGE of isolated aTnC (mixture 60:40 with native cTnC, see Methods; lanes 7 and 8) and native cTnC (lanes 9 and 10). Denaturation conditions were either non-reducing (no DTT; lanes 7 and 9) or reducing (2 mM DTT; lanes 8 and 10). The migration of aTnC and native cTnC were indistinguishable under identical conditions, indicating that aTnC does not result from either proteolysis or dimerization. For both native cTnC and aTnC, the differential migration between reducing versus nonreducing conditions may reflect either that: (a) both migrate similarly under the same conditions; or (b) each form migrates differently under the same condition and that there is interconversion between the two forms under the conditions of the gel.

1). Incorporation of aTnC was judged to be complete when the force reached a steady level (about 10 minutes).

SDS-PAGE verified that aTnC was incorporated into TnC extracted fibers, as is native cTnC (Fig. 3 A) (8, 27–30). Because SDS-PAGE indicated that the fiber content of neither TnT nor TnI was altered by the extraction/reconstitution procedure (Fig. 3 A), the force resulting from aTnC incorporation did not result from loss of whole troponin (31, 32).

Force resulting from reconstitution with aTnC was found to be unchanged by elevated  $[Ca^{2+}]$  (Fig. 1, 2), and was approximately equal to the maximum  $Ca^{2+}$  activated force of fibers reconstituted with native cTnC (Fig. 2; fibers with native cTnC generate no active force in the absence of  $Ca^{2+}$ ). These results demonstrate that aTnC enables thin filament activation without  $Ca^{2+}$  and thus supports the hypothesis that the structure of aTnC has important features in common with the  $Ca^{2+}$ -bound state of unmodified cTnC.

Two observations indicate that the mechanism of force generation in fibers activated by aTnC was the same in  $Ca^{2+}$  activated fibers with unmodified TnC. First, aluminofluoride, a structural analog of orthophosphate which requires active crossbridge cycling to be effective (18), inhibited aTnC activated force production (Fig. 1). Additionally, fibers reconstituted with aTnC shortened actively and redeveloped force following a period of unloaded shortening. In aTnC activated fibers,

unloaded shortening velocity ( $V_{us}$ ) measured by the slack method (33) was the same in the presence and absence of  $Ca^{2+}$  ( $1.50 \pm 0.22 \text{ FL} \cdot \text{s}^{-1}$  at pCa 4 versus  $1.50 \pm 0.26 \text{ FL} \cdot \text{s}^{-1}$  at pCa 9.2;  $N = 14$ ; paired t-test,  $P > 0.05$ ) and was also the same as  $V_{us}$  with native cTnC at pCa 4 ( $1.28 \pm 0.31 \text{ FL} \cdot \text{s}^{-1}$  at pCa 4;  $N = 4$ ; t-test,  $P > 0.05$ ). Control  $V_{us}$  prior to extraction of native sTnC was  $2.54 \pm 0.35 \text{ FL} \cdot \text{s}^{-1}$  ( $N = 18$ ).

The ability of aTnC to activate force without  $Ca^{2+}$  allows design of experiments to directly test whether  $Ca^{2+}$  affects contraction through proteins other than TnC (5). For example, the observation that  $V_{us}$  was insensitive to  $Ca^{2+}$  in aTnC activated fibers suggests that  $Ca^{2+}$  binding to sites other than TnC does not directly modulate actomyosin kinetics during shortening. This contrasts with a report that  $Ca^{2+}$  binding to myosin light chain 2 may modulate the kinetics of isometric force development (34). Activation by aTnC contrasts with other procedures which result in force in the absence of  $Ca^{2+}$  such as (a) lowering  $[MgATP]$  (35) which impairs active shortening (36, 37), (b) extraction of whole troponin, which results in neither complete extraction nor complete activation (31, 32), and (c) activation in solutions of low  $[Mg^{2+}]$  and low ionic strength which might alter the function of other proteins (38).

Partial extraction of sTnC from fibers (Fig. 4 A) (39), followed by reconstitution with aTnC (Fig. 4 B), resulted in maximum attainable force consisting of  $Ca^{2+}$  sensitive and insensitive components (Fig. 4 B). This

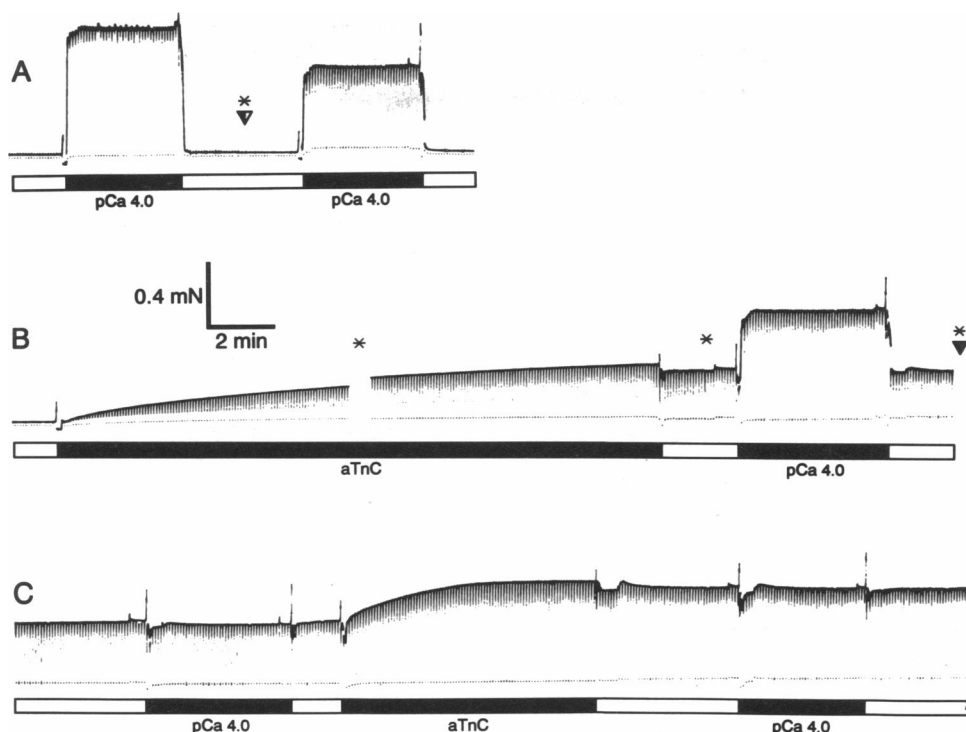


FIGURE 4 Force recorded during partial sTnC extraction (A), partial reconstitution with aTnC (B), and full reconstitution with aTnC subsequent to selective extraction of residual sTnC (C). The record is discontinuous at the times indicated by (\*). Periods of exposure to  $\text{Ca}^{2+}$  (pCa 4.0), TnC extraction (inverted triangles), and reconstitution with aTnC ( $0.22 \text{ mg} \cdot \text{ml}^{-1}$ ) are indicated as in Fig. 1. A. Partial sTnC removal was effected by abbreviated extraction (2 min); the extent of extraction was evidenced by decreased  $\text{Ca}^{2+}$  activated force at pCa 4.0. B. During partial reconstitution with aTnC, force rose to a steady but submaximal level. At pCa 4.0, force rose to 87% of control, revealing coexistence of  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent regulation. C. Subsequent re-exposure to extracting solution resulted in removal of only the  $\text{Ca}^{2+}$ -sensitive component of force (residual sTnC). Finally, full reconstitution with  $0.22 \text{ mg} \cdot \text{ml}^{-1}$  aTnC resulted in maximum  $\text{Ca}^{2+}$ -insensitive force.

result indicates that the degree of activation of force by aTnC is related to the number of available TnC binding sites on the thin filament. Significantly, when fibers containing both sTnC and aTnC were exposed to extracting solution for a second time, only sTnC was removed, as indicated by the removal of only the  $\text{Ca}^{2+}$  sensitive component of force (Fig. 4 C). Qualitatively similar results were obtained by fully extracting endogenous sTnC (as in Fig. 1) and using a mixture of aTnC and native cTnC for the first reconstitution. These results suggest that aTnC interacts strongly with the other thin filament regulatory proteins in the absence of divalent cations, and is consistent with the hypothesis that the interaction between TnC and Tnl is strengthened during activation (12).

aTnC probably has an intramolecular disulfide bond between Cys 35 and Cys 84. From force measurements, we found that the calcium sensitivity of aTnC could be restored by treatment of isolated aTnC with 5 mM DTT under denaturing conditions, followed by renaturation (see Methods). This result indicates that structural changes resulting from reversible disulfide bond formation enable aTnC to activate force generation in skinned fibers. Furthermore, SDS-PAGE of the isolated protein run under non-reducing conditions indicated that aTnC

was not a dimer resulting from intermolecular disulfide bonds (Fig. 3 B), in agreement with Putkey et al. (16). To further verify the presence of a disulfide bond in aTnC, we measured the relative numbers of free cysteine residues in aTnC versus native cTnC. Because denaturation of aTnC under reducing conditions (5 mM DTT) restored  $\text{Ca}^{2+}$  sensitivity, we did not incubate the proteins with urea/DTT prior to determination of free cysteines (22, 40). For a preparation of aTnC which was judged to be 70% aTnC and 30% cTnC by force measurements in skinned fibers, there were only about  $0.35 \pm 0.21$  ( $N = 4$ ) as many cysteines in the mixture as found for native cTnC, compared with an expected ratio of 0.3 if aTnC had no free cysteines. The lower number of free cysteines in the aTnC mixture is consistent with the presence of a disulfide bond in aTnC as suggested by Putkey et al. (16). It was not possible to reduce the disulfide bond with 5 mM DTT under normal experimental conditions (i.e., non-denatured), either in solution or with aTnC reconstituted into fibers. This indicates that the disulfide bond is stable under normal physiological salt conditions and relatively inaccessible to conventional reducing agents.

While our results are consistent with intramolecular crosslinking between Cys 35 and Cys 84, it is possible

that force generation with aTnC resulted from modification at site I alone, since Cys 35 is within the inactive site I in cTnC. However, site-directed mutagenesis in which site I of cTnC was modified to bind  $\text{Ca}^{2+}$ , resulted in no additional force generation in slow skeletal fibers (8). In addition, a second point mutation to inactivate site II resulted in a complete loss of  $\text{Ca}^{2+}$  activated force, even though the isolated protein was demonstrated to bind  $\text{Ca}^{2+}$  and to be incorporated into fibers (8). Thus, these observations imply that modification at site I is not sufficient to activate contraction.

The modification of cTnC studied here, which resulted in a functionally activated molecule, contrasts with other structural modifications at site II of TnC which resulted in functionally inactivated molecules. The conformational changes which are thought to accompany  $\text{Ca}^{2+}$  binding to site II in sTnC (9, 11) were inhibited by interactions between mutated residues in site II and the D helix (13, 14). Our observations in skinned fibers indicate that intramolecular crosslinking between site I and the D helix in the linker region of cTnC resulted in conformational changes within the molecule, presumably at site II, that mimic those induced by calcium binding to native cTnC, and support the hypothesis that the structure of aTnC has important features in common with the  $\text{Ca}^{2+}$ -bound state of unmodified cTnC.

In summary, we found that a reversible modification of rabbit cTnC results in a molecule which, when reconstituted into skinned skeletal muscle fibers, enables force production and shortening in the absence of  $\text{Ca}^{2+}$  (pCa 9.2). When only aTnC was present in fibers, force and  $V_{\text{us}}$  were insensitive to elevated  $\text{Ca}^{2+}$  (pCa 4.0), indicating that  $\text{Ca}^{2+}$  does not affect these mechanical parameters by binding at sites other than TnC. Thus, aTnC may serve as a stable structural model for calcium induced conformation changes in native cTnC, a necessary but missing component needed to test current models of structure-function relationships in troponin regulation of muscle contraction.

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